REMARKS

Reconsideration of the captioned application in light of the foregoing amendments and following comments is courteously requested.

Claims 1-10 are pending. Claims 1-10 stand rejected. Claims 1-10 are objected to. Claims 1, 2, 4, 5, 6 and 7 have been amended. Claim 1 has been amended to provide proper antecedent basis and to provide a correlating step. Claim 4 has been amended to provide proper antecedent basis. Claim 7 has been amended to point out the recited resin as "capture resin". Clam 2, 4, 5 and 6 have been amended to provide proper Markush type language to claims. The amended claims are attached as "Version with markings to show changes made".

OBJECTION TO THE ABSTRACT

The Examiner has objected to the language and format of the abstract. A new abstract on a separate page is provided containing corrections to address "...proper language and format for an abstract of the disclosure" in order to overcome the objection. The abstract provided is supported by the specification and thus no new matter is added. Applicants believe the new abstract is in compliance with the rules and therefore request that the objection be withdrawn.

OBJECTION TO THE DRAWINGS

The Examiner has objected to the drawings because of insufficient labeling of Figure 3. The Examiner cites that "...the plate in the figure does not disclose the contents of each well nor does it disclose any numerical data, thus interpretation of results is not possible." Applicants believe that Figure 3 is sufficiently labeled and supported by the specification.

For example, the description of Figure 3 states that the reaction according to the method of Example 1 takes place in each well in the presence of "a test compound" and

further refers to " test compound from a chemical library". In the specification a "solution of a test compound" used in the experiment of Example 1 "of a compound plate" is described as "a polypropylene surface having a multiplicity of compartments into which 1μl of a 1mM solution of a test compound in dimethyl sulfoxide (DMSO) is placed" (paragraph 49, lines 1-3). The description of Figure 3 states "[f]igure 3 shows the results from performing FabD assays in individual wells of a 96-well microplate according to the method described in Example 1. The FabD reaction takes place in each well in the presence of a test compound from a chemical library" (paragraph 24).

In addition, the contents of each well of Figure 3 are further described in the specification, Example 1, "[n]inety-six organic molecules (test compounds) were obtained from a chemical library and dissolved in DMSO to a concentration of 1 mM. Each of the solutions of test compounds were added to individual compartments of a polypropylene 96-well plate" (paragraph 75).

A description providing sufficient support for Figure 3 plate results is found in Example 1 of the specification (paragraph 75, lines 14-20) stating "[c]ompartments exhibiting high radioactivity (approximately equal to that of control compartments containing no test compounds or enzyme inhibitors) contained test compounds that are inactive at inhibiting the FabD enzyme. Compartments exhibiting less radioactivity compared to the control compartment contain test compounds that were effective at inhibiting the catalytic effects of the FabD enzyme. The results of the foregoing assay are depicted in Figure 3." The description of Figure 3 describes "FabD activity measured in each well is compared to that of control wells (containing no test compounds) and is reported as percent inhibition. Levels of percent inhibition of FabD activity in each well is represented by shading. A black shaded dot represents a well with 50 to 100% reduction of FabD activity in the presence of a test compound. A gray shaded dot represents a well with 0-50% reduction in FabD activity in the presence of a test compound." (paragraph 24). Thus the specification discloses numerical data by way of shaded dots that represent the range of percent activity of the control reaction, and the specification adequately describes the contents of each well. Therefore Applicants assert

that Figure 3 is adequately labeled and request that the objection to the drawing be withdrawn.

35 U.S.C. §112, FIRST PARAGRAPH

The Examiner rejected Claims 1-10 under 35 U.S.C. §112, first paragraph as allegedly containing subject matter that is not enabling. The Examiner states that "[Example 1 and Figure 3 are] not enabling for the claimed invention because results shown for a single assay are meaningless where one does not know their accuracy. No inhibitors of any enzymes are taught in the specification as originally filed." Applicants respectfully disagree and believe that the claimed invention is enabled by the disclosure.

Example 1 and Figure 3 are presented as a working example of the claimed method, which demonstrates that inhibitors of a dual substrate inhibitor can be identified by a method of the present invention. In context with the full disclosure of the instant specification, Applicants submit the claimed invention is fully enabled. The Examiner's focus on the data disclosed in Figure 3 is misplaced, rather Figure 3 should be viewed in the context of the disclosure as a whole. Support for the Applicants position that the claimed invention is fully enabled is provided below.

Claims of the present application are directed to a method for identifying inhibitors, not to the inhibitors. As the Examiner correctly states "[c]laims 1-10 are directed to a method for identifying an inhibitor of a dual substrate enzyme" (page 3, paper 5). Knowledge of an inhibitor of a dual substrate enzyme is not required to practice the method of the present invention. One skilled in the art reading the disclosure of the present application would be enabled to make and use the methods as claimed to identify inhibitors of dual substrate enzymes.

One skilled in the art would know how to select compounds for use as test compounds in the methods of the present invention. For example, one of skill in the art would recognize chemical libraries as a source of test compounds. Chemical libraries and how to make them are well known in the art. For example, WO 98/20349

(demonstrates the preparation of novel chemical libraries), WO 96/21859 (discloses "...catalogued chemical libraries containing a multiplicity of reaction products and that are useful for screening for a variety of uses including for pharmacological activity") and WO99/54031 (discloses methods for the synthesis of organic compound libraries). Thus, one skilled in the art would know how to select test compounds for use in the present invention. Applicants point out that the use of a compound in the present invention does not require prior knowledge of an inhibitor of the dual substrate enzyme. The method of the present invention identifies which test compounds are inhibitors. Example 1 data shown in Figure 3 demonstrates that the method of the present invention can be used to select inhibitors from compounds obtained from a chemical library.

Example 1 describes screening of test compounds from a chemical library. Figure 3 shows the results from performing FabD assays in individual wells of a 96-well microplate according to the method described in Example 1. These data show that from screening compounds from a library, at least four FabD inhibitors were found based on the ability of these compounds to reduce FabD activity 50-100% (represented by a black shaded dot) compared with control.

While prior knowledge of an inhibitor of the dual substrate enzyme is not necessary to use the method of the present invention, one skilled in the art would be able to identify standard dual substrate enzyme inhibitors disclosed in the art. One skilled in the art would know how to obtain and use such compounds as positive controls in the method of the present invention. For example, Joshi et al, Archives of Biochemistry and Biophysics 143, 493-505 (1971), discloses that both CoA and Acetyl CoA are inhibitors of FabD. He et al., Analytical Biochemistry 282, 107-114 (2000), disclose that iodoacetamide and thiolactomycin are inhibitors of FabH. Copies of these references are attached for the convenience of the Examiner.

Applicants submit that the specification, as filed, enables one of skill in the art to make and use the claimed methods. CoA is disclosed in the present specification (paragraph 0034, line 2) and as discussed above is a well known inhibitor of FabD as

disclosed by Joshi. To further support that the present invention is enabled, and to address the Examiner's statement regarding the accuracy of the data, Applicants have attached supporting data, which show dose dependent inhibition of FabD activity by CoA. These data demonstrate that inhibition activity of CoA can be identified by the claimed method. These data demonstrate that methods of the claimed invention can accurately produce an inhibition curve of FabD activity duplicative of the known pharmacology of CoA, thus validatating these methods. Furthermore, these data show that the methods of the present invention provide data over a range of inhibitor concentrations in a dose dependent manner, an indication of a high level of confidence in the data. Thus, one of skill in the art would be able to make and use the claimed invention to test compounds and identify novel chemical compounds as inhibitors of a dual substrate enzymes. Additionally, as discussed above, the results shown in Figure 3 of the present invention demonstrate that the claimed invention can be used to identify inhibitors of a dual substrate enzyme. Thus, the specification, as filed, enables one skilled in the art to accurately identify whether a test compound is an inhibitor of a dual substrate enzyme according to the methods of the present invention.

For the reasons given above, Applicants assert that Claims 1-10 are enabled and request withdrawal of the rejection of Claims 1-10 under 35 U.S.C. §112, first paragraph.

35 U.S.C. §112, SECOND PARAGRAPH

The Examiner has rejected Claim 1 under 35 U.S.C. §112, second paragraph for reciting the limitations "the radiolabeled portion", "the non-radiolabeled first substrate", "test compound" and "the enzyme-radiolabeled first substrate" in lines 3, 7, 9 and 11, respectively. The Examiner states that there is insufficient antecedent basis for these limitations in the claims. Claim 1 has been amended to replace "the" with "a" or "an" for the limitations in lines 3, 7 and 11. Applicants believe "a test compound" in line 8 has sufficient antecedent basis. However, Applicants have amended line 14 to replace "a" with "the".

The Examiner has rejected Claim 1 under 35 U.S.C. §112, second paragraph as being incomplete "for omitting essential steps, such omission amounting to a gap between the steps." Claim 1 has been amended to include a correlating step. Support for this amendment is found in the specification, paragraph 75, lines 14-19 therefore no new matter has been added.

The Examiner has rejected Claim 4 under 35 U.S.C §112, second paragraph for insufficient antecedent basis for "the enzyme". Claim 4 has been amended to depend from Claim 1 rather than Claim 3. Thus, amended Claim 4 has sufficient antecedent basis.

Claim 7 stands rejected under 35 U.S.C. §112, second paragraph, because "the claim fails to particularly point out whether the recited resin refers to the 'capture resin' or the "'scintillant resin' in Claim 1." Applicant's have amended Claim 7 to clarify that the "resin" refers to the "capture resin" of Claim 1.

The Examiner has rejected claims 2, 3-4 and 6 for improper Markush language. Claims 2, 4, and 6 have been amended to omit "selected from". Applicants believe that Claim 3 is in proper claim format, but note that Claim 5 is not. Claim 5 has been amended to omit "selected from".

Applicants believe that all the claims are now in proper claim format and request withdrawal of the rejections of the Claims under 35 U.S.C. §112, second paragraph.

35 U.S.C §102(e)

Claims 1-4 and 8 are rejected under 35 U.S.C. §102 (e) as allegedly being anticipated by Reynolds et al., (U.S. Patent No. 20010031477 A1) with the Examiner citing "...page 1, abstract; pages 2-3, paragraphs 11-12, 14-17; page 4, paragraphs 43; page 5, paragraph 49 and 54; page 8, claims 1 and 7." Applicants respectfully traverse the rejection and courteously submit that the invention embraced by Claims 1-4 and 8 are

not anticipated by Reynolds within the meaning of 35 U.S.C. §102(e). To anticipate a claim the reference must disclose each and every element of the claim. MPEP 2131

The instant claims are directed towards methods for identifying inhibitors of dual substrate enzymes, comprising adding a capture resin to the reaction mixture and removing unreacted radiolabeled second substrate from the reaction mixture and adding a scintillant resin. The present invention uses no ligand tagging of the first substrate, therefore no potential deleterious modification is necessary. First substrate is combined with the radiolabeled second substrate and reacted in the presence of enzyme in the presence or absence of a test compound. A capture resin is used to bind the radiolabeled first substrate (product), and the unreacted radiolabeled second substrate is removed (e.g. by filtration). Scintillant resin is then added to the first substrate mixture and the amount of radiolabeled first substrate is measured. The present invention does not rely on product binding to the scintillant resin or on the dilution of the reaction mixture but rather the co-mingling of the capture beads and scintillant beads after removing unreacted second substrate.

Reynolds discloses a method to assay enzymatic activity of bacterial fatty acid biosynthesis enzymes. Reynolds' method involves reacting an enzyme with a ligand tagged substrate and a radiolabeled second substrate. The reaction mixture is then exposed to a SPA support system (e.g. a bead) in which scintillant is trapped and to which receptor molecules are attached. (Reynolds, paragraphs 0011 and 0040). Scintillation occurs when a weak energy radioactive isotope such as ^{3}H is brought sufficiently close to the SPA support system. This occurs through the binding of the ligand portion of the radiolabeled reaction product to the beads. Reynolds notes that "[i]n the practice of the present invention the use of SPA obviates the need for separation of radioactive product from the radioactive substrate." (paragraph 0040, lines 16-18). Reynolds further states that "[a]ssays in SPA format do not involve cumbersome washing steps." (paragraph 40, lines 20-21). Reynolds relies on the ligand binding to the receptor/SPA support to bring the radioactive reacted product into scintillation proximity

with the scintillant. Reynolds relies on dilution to prevent the unreacted radiolabeled substrate from exciting the SPA resin.

A required element of Claims 1-4 and 8 is a separation step removing unreacted radiolabeled second substrate from the reaction mixture. The Examiner is correct in stating "Reynolds' invention does not include a separation step for the product and residual substrate" (page 7, lines 5-6, paper 5). Reynolds does not have a separation step that involves removing unreacted radiolabeled substrate from the reaction mixture. Therefore Reynolds does not disclose a required element of the claimed invention. Reynolds relies on the use of a tagged substrate binding to receptors which are bound to the scintillant resin. Reynolds specifically discloses that "[in] the practice of the present invention, the use of SPA obviates the need for separation of a radioactive product from the radiolabeled substrate." (Reynolds, Paragraph 40). Reynolds retains the unreacted radiolabeled substrate in the mixture solution. Thus, Reynolds fails to disclose the required element of a separation step removing unreacted radiolabeled second substrate from the reaction mixture.

Reynolds does not disclose the use of two separate resins as required in the present invention. One element of the claims is adding a capture resin. Another element of the claims is adding a scintillant resin. Reynolds does not disclose adding a scintillant resin that is physically separate from the means for immobilizing the radiolabeled radioactive product. Reynolds relies on the ligand binding to the receptor/scintillant resin and clearly does not use two separate resins as required in the method of the present invention. In contrast, the present invention requires the separate use of a capture resin used to retain the radiolabeled product and the separate use of a scintillant resin.

Because Reynolds fails to disclose all the elements of the claimed invention, rejection of Claims 1-4 and 8 under U.S.C. 102 (e) is therefore in error and should be withdrawn.

35 U.S.C. §103(a)

Claims 5 and 6 stand rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over Reynolds in view of Mathews (Mathews, C.K., and van Holde, K.E., Biochemistry-Second Edition, 1995). Applicants respectfully traverse the rejection and courteously submit that the invention embraced by Claims 5 and 6 is not prima facie obvious within the meaning of 35 U.S.C. §103(a).

Establishing a prima facie case of obviousness requires:

1. some teaching or suggestion to one of ordinary skill in the art to modify the reference or to combine reference teachings.
2. a reasonable expectation of success
3. that the references when combined must teach or suggest all the claim limitations. MPEP 2142

The Examiner states "Mathews teaches that fatty acid synthesis is controlled in part by phosphorylation and dephosphorylation, i.e. via kinases and phosphatases, (page 654), thus qualifying the enzymes in claims 5 and 6 as enzymes involved in the fatty acid biosynthetic pathway." The Examiner concludes "[t]herefore, it would have been obvious at the time the invention was made to a person having ordinary skill in the art to modify the assays as disclosed by Reynolds for use with phosphate transfer enzymes." Applicants respectfully disagree.

The disclosure of Reynolds is discussed in detail above.

Mathews discloses a biochemical overview of the fatty acid biosynthetic pathway.

There is no suggestion or motivation for one of skill in the art to modify the references to arrive at the present invention. Even if one skilled in the art were to combine the references, they would not teach or suggest all the claim limitations of the present invention. In fact, the primary reference teaches away from the modifications of the methods it discloses which would be necessary to arrive at the present invention.

The Examiner implies that the method of Reynolds is the same as the claimed invention except that it does not specifically disclose phosphate transfer enzymes. Applicants respectfully disagree. As discussed above under the 102(e) rejection, Reynolds does not disclose all of the elements of the present invention. Not only does Reynolds fail to disclose use of a separation step (i.e. removing unreacted radiolabeled second substrate) and use of a capture resin separate from the scintillant resin, but Reynolds teaches away from the present invention.

The present invention requires a separation step to remove unreacted radiolabeled second substrate from the reaction mixture. Reynolds does not disclose the separation step of the present invention. Rather than separating the unreacted radiolabeled substrate from the reaction mixture as required by the present invention, Reynolds relies on dilution of the reaction mixture solution to prevent the unreacted radiolabeled substrate from exciting the SPA resin. Reynolds teaches away from the separation step of the present invention by retaining the unreacted radiolabeled substrate in the assay mixture, stating "[t]he unreacted ^{3}H acetyl-CoA substrate remains in solution and is sufficiently distant from the scintillant that no significant signal is observed." (paragraph 0047) Reynolds points out the advantages of the method disclosed, specifically reciting that "the use of SPA obviates the need for separation of a radioactive product from the radiolabeled substrate" and that the "[a]ssays in SPA format do not involve any cumbersome washing steps" paragraph 40, lines 16-21 . By presenting the advantages of not having a separation step, Reynolds teaches away from including a separation step, which is a limitation of the present claims. Mathews does not make up for this deficiency of Reynolds. Mathews does not teach or suggest removing an unreacted radiolabeled second substrate from a reaction mixture. Mathews simply discloses that the fatty acid synthetic pathway is controlled in part by phosphorylation and dephosphorylation.

Claims 5 and 6 also include a limitation of adding a capture resin and a limitation of adding a scintillant resin. As discussed above, Reynolds uses one resin to function as a capture resin and as a scintillation resin. Reynolds discloses a "Scintillation Proximity Assay (SPA) support system, where the SPA support system is coated with receptors

having high affinity for the ligand moiety of the conjugate; and measuring the radioactivity attached as a result of this specific ligand-receptor interaction" (paragraph 0017). Reynolds relies on the receptor being bound to the SPA resin in order to bring the radiolabeled product into proximity of the scintillant in a diluted mixture. Reynolds thus teaches away from using separate beads to bind the product and to contain the scintillant as that would not ensure the close proximity of radiolabeled product and scintillant required in an SPA. Mathews does not teach or suggest a method for using capture resin separate from a scintillant resin in assays of dual-substrate enzymes. Mathews simply discloses that the fatty acid synthetic pathway is controlled in part by phosphorylation and dephosphorylation. Thus, Mathews does not make up for the deficiency of Reynolds.

For the reasons above, neither Reynolds nor Mathews, alone or in combination, teach or suggest all of the limitations of the claimed invention. Reynolds teaches away from modifying the methods in Reynolds to include a separation step or to separately add a capture resin and scintillant resin. And Mathews suggests nothing in regard to modifying such a method. Because the Examiner has failed to state a prima facie case of obviousness, the rejection of Claims 5 and 6 under 35 U.S.C. §103(a) is in error and should be withdrawn.

Claims 7 and 9 and 10 are rejected under 35 U.S.C. §103(a) as being unpatentable over Reynolds in view of Gul et al., (Enzyme Assays-Essential Data, 1998). Applicants respectfully traverse the rejection and courteously submit that the invention embraced by Claims 7, 9 and 10 is not prima facie obvious within the meaning of 35 U.S.C. §103(a).

The Examiner correctly states that "Reynolds' invention does not include a separation step" and that "Reynolds recites that, the use of SPA [Scintillation Proximity Assay] obviates the need for separation." The Examiner further states that Gul teaches "[t]he success of a radiometric assay relies on the efficient separation of the product formed during the reaction from residual substrate (either which may be radiolabeled) and its sensitivity is dependent upon the specific radioactivity of the compound." The Examiner cites Gul as teaching various separation techniques and concludes "it would

have been obvious...to modify Reynolds' invention by including a separation step so as to increase the sensitivity of the assay." Applicants respectfully disagree.

The method of Reynolds is discussed in detail above.

Gul is a reference text on enzyme assays. Gul describes radiometric assays in general (section 3.4, page 38) and in a separate section discusses scintillation proximity assays (SPA) (section 3.8, page 40). For general radiometric assays, Gul teaches the need for a separation step stating "[t]he success of a radiometric assay relies on the efficient separation of the product formed during the reaction from residual substrate"(page 37). For general radiometric assays, Gul teaches various separation techniques (pages 38-39) However, in the section devoted to scintillation proximity assays, Gul teaches that a separation step is not used or needed in a SPA assay. Gul teaches that "the scintillant proximity assay (SPA), has the major advantage that the physical separation of product from substrate is not required" (page 37). Furthermore, Gul teaches that only one resin is used in a SPA assay. Gul recites that SPAs use "microsphere beads that are embedded with a scintillant and coated with a specific capture molecule" (section 3.8, page 40).

The Examiners' rejection implies that the method of Reynolds is the same as the present invention except that it does not contain a separation step, and when combined with Gul, would lead one skilled in the art to arrive at the claimed method. This is not the case. There is no suggestion or motivation in Reynolds or Gul to use a separation step or to use a capture resin separately from a scintillant resin in a SPA. Both Reynolds and Gul teach away from modifying a SPA to include the three limitations that are required elements of the claimed invention.

As discussed in detail above, the method of the claimed invention requires a separation step whereas Reynolds does not include a separation step. Reynolds teaches away from including a separation step. The Examiner states in the rejection of Claims 7, 9 and 10 that "it would have been obvious to a person having ordinary skill in the art at the time the invention was made to modify Reynolds' invention by including a separation

step so as to increase the sensitivity of the assay." Applicants respectfully disagree. There is no suggestion in Reynolds or Gul that one would want or need to increase the sensitivity of a SPA. Thus, the references cannot provide motivation to modify Reynolds for increasing sensitivity. Nor is there any other suggestion or teaching in either reference that provides motivation to add a separation step.

The Examiner points to the introductory section of Gul which teaches that it is desirable to use a separation step in general radiometric assays by referring to the section of the disclosure that teaches, "[t]he success of a radiometric assay relies on the efficient separation of the product formed during the reaction from residual substrate". However, the Examiner has failed to appreciate the context in which Gul teaches a separation step. One of skill in the art reading the entire introduction would recognize that Gul specifically distinguishes between the more convenient radiometric scintillation proximity assays and radiometric assays in general. The statement from Gul cited by the Examiner was referring to a general radiometric assay, not a SPA. Therefore, Gul, when taken as a whole, teaches that a separation step is not necessary or desirable when using SPAs. In section 3.8 which describes SPAs, Gul teaches that in a SPA in "aqueous solution, if a radiolabeled compound is unbound its energy is dissipated and fails to excite the scintillant of the bead. However, if the radiolabeled compound is bound to the bead [containing the scintillant] via the capture molecule, higher counts are observed" (section 3.8, page 40). Gul concludes that "the scintillation proximity assay (SPA), has the major advantage that the physical separation of product from substrate is not required" (section 3.1, page 37). Thus, Gul teaches away from using a separation step in a scintillation proximity assay, a limitation of the present invention. Therefore, one of skill in the art viewing the disclosure of Gul as a whole, would not have been motivated to modify the SPA method of Reynolds to include a separation step and arrive at the claimed invention.

Claims 7, 9 and 10 include the limitations of adding a capture resin and adding a scintillant resin. As discussed in detail above, Reynolds does not disclose each and every element of the present invention. Reynolds does not disclose the addition of a capture

resin and the separate addition of scintillation resin, limitations of the present invention. Reynolds requires that the scintillant resin also function as a capture resin.

In the context of SPAs, Gul explains that the radioactive product must be bound to the scintillant bead "via the capture molecule". Gul teaches use of one bead as a scintillant resin and capture resin for detection of reacted radiolabeled product, stating in SPAs in "aqueous solution, if a radiolabeled compound is unbound its energy is dissipated and fails to excite the scintillant of the bead. However, if the radiolabeled compound is bound to the bead [containing the scintillant] via the capture molecule, higher counts are observed" (section 3.8, page 41). Gul thus teaches away from the use of separate capture and scintillant resins. Like Gul, Reynolds uses one resin as a capture/scintillant resin where "the SPA support system is coated with receptors" to capture a ligand. Both Reynolds and Gul, in the context of discussion of SPAs, focus on the importance of the scintillant containing resin also having a capture molecule attached. Thus, one of skill in the art would not have been motivated by the disclosure of Gul to modify Reynolds to arrive at the claimed methods.

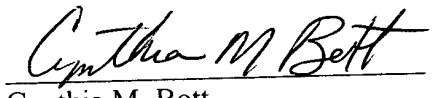
In conclusion, one having ordinary skill in the art at the time the invention was made would not have been motivated to modify Reynolds' method in view of Gul to include a separation step or separate steps of adding a capture resin and a scintillant resin to arrive at the present invention. Because the Examiner has failed to state a prima facie case of obviousness, the rejection of Claims 7, 9 and 10 is in error and should be withdrawn.

Summary

Applicants have properly stated, traversed, accommodated, or rendered moot each of the Examiner's grounds for rejection. Applicants submit that the present application is now in condition for allowance.

Applicants courteously request the Examiner to reconsider the application in light of the foregoing comments, to withdraw the rejection of Claims 1-10 and to pass the case to issue.

Respectfully submitted,



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Dated: February 21, 2003

Attachments - Amended claims version with markings to show changes made
Abstract on a separate page
Data-Inhibition of FabD by CoA
Copy of Joshi et al.
Copy of He et al.

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE CLAIMS:

1. (AMENDED). A method for identifying an inhibitor of a dual substrate enzyme; wherein a first substrate is a macromolecule that is enzymatically modified, in the presence of the dual substrate enzyme, to accept [the] a radiolabeled portion of a second substrate, said method comprising:

   a. adding a capture resin to a buffered mixture of an enzyme, allowing the enzyme to catalyze transfer of the radiolabeled portion of the radiolabeled second substrate to [the] a non-radiolabeled first substrate,[radiolabeled first substrate, and a radiolabeled second substrate,] in the presence or absence of a test compound;

   b. removing unreacted radiolabeled second substrate;

   c. adding a scintillant resin to [the] an enzyme-radiolabeled first substrate mixture; [and]

   d. measuring the amount of radiolabeled first substrate reacted in the presence of [a] the test compound by scintillation counting, measuring the amount of radiolabeled first substrate reacted in the absence of [a] the test compound by scintillation counting, and comparing the two measurements[.] ; and

   e. wherein when the amount of reacted first substrate is lower in the presence of a test compound than in the absence of the test compound, the test compound is identified as an inhibitor.

2. (AMENDED). A method according to Claim 1 wherein the first substrate is a [macromolecule selected from a] peptide or protein.

4. (AMENDED) A method according to Claim [3] 1 wherein the enzyme is [selected from] a fatty acid biosynthesis enzyme.

5. (AMENDED) A method according to Claim 1 wherein the enzyme is [selected from] a phosphate transfer enzyme.

6. (AMENDED) A method according to Claim 5 wherein the enzyme is [selected from] a protein kinase or protein phosphatase enzyme.

7. (AMENDED) A method according to Claim 1 wherein the capture resin is an ionically charged resin.

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Studies on the Mechanism of Fatty Acid Synthesis

XXVI. Purification and Properties of Malonyl-Coenzyme A—Acyl Carrier Protein Transacylase of *Escherichia coli*[1]

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Malonyltransacylase, purified 950-fold from extracts of *Escherichia coli*, was found to be homogeneous by disc-gel electrophoresis. The molecular weight of the enzyme was estimated to be 35,500 daltons, based upon polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. The enzyme, while insensitive to sulfhydryl inhibitors, is strongly inactivated by phenylmethanesulfonylfluoride, suggesting that it possesses an active serine residue. The transacylase accepts the malonyl group from malonyl-CoA or malonyl-ACP to form a malonyl-enzyme intermediate. This intermediate is stable to performic acid oxidation; on hydrolysis with pepsin it yields peptic peptides also stable to performic acid.

The malonyl group from malonyl-enzyme intermediate can be transferred to ACP, CoA, pantetheine, *N*-(*N*-acetyl-β-alanyl) cysteamine, or *N*-acetylcysteamine. The enzyme is specific for the malonyl group and does not transfer acetyl group from acetyl-CoA. But acetyl-CoA is a competitive inhibitor of malonyl-CoA, with a K_i value of 115 μM.

Data from kinetic studies of the transacylase reaction are consistent with the formation of a malonyl-enzyme intermediate during the reaction. Lineweaver-Burk plots at different concentrations of malonyl-CoA and at various fixed concentrations of ACP give a series of parallel lines. Moreover, CoA is found to be a competitive inhibitor of ACP, and malonyl-ACP a competitive inhibitor of malonyl-CoA. These findings are consistent with a Ping-Pong Bi Bi kinetic scheme for the malonyl-transacylase reaction.

The synthesis of long-chain fatty acids in *Escherichia coli* occurs by way of acyl intermediates bound in thioester linkage to a low molecular-weight heat-stable protein, acyl carrier protein (2-5). The enzymes catalyzing the six intermediate reactions for the fatty acid biosynthesis have been separated from each other and purified from crude extracts of *E. coli* (6). Partially purified preparations of acetyl-CoA-ACP[2] transacylase and malonyl-CoA-ACP transacylase have been isolated from *E. coli* and their properties investigated (4, 7). Both enzymes were reported to be sulfhydryl enzymes because of their inhibition by sulfhydryl-binding agents.

In contrast to the readily dissociable synthetase from *E. coli*, the fatty acid synthetases from pigeon liver and yeast are isolated as tightly bound complexes. Studies from

[1] This investigation was supported in part by Grant GM-06242-12 from the National Institutes of Health, United States Public Health Service, by a grant from the American Cancer Society, and Grant 70-1030 from the American Heart Association, Inc. The preceding paper of this series is Ref. 1.

[2] The abbreviations used are: ACP, acyl carrier protein; E, Enzyme; E-mal or E-*o*-mal, malonyl-enzyme intermediate; mal-CoA, malonyl-CoA and mal-ACP, malonyl-ACP.

this laboratory on the acetyl and malonyltransacylase activities of pigeon fatty acid synthetase had suggested that the nonthiol binding site of the synthetase was involved in the transacylation of acyl groups to ACP (8, 9). This finding prompted us to reexamine the acyltransacylases of *E. coli*. In this paper we report the isolation of a homogeneous preparation of malonyltransacylase from *E. coli*. The enzyme is not a sulfhydryl enzyme and requires the participation of a nonthiol group in its activity. It catalyzes the reaction by forming a covalent linkage with the malonyl group at the nonthiol binding site. Kinetic studies of the enzyme are consistent with a Ping-Pong Bi Bi mechanism.

MATERIALS AND METHODS

Acetyl-CoA and CoA were obtained from P-L Biochemicals, Inc.; dithiothreitol, phenylmethanesulfonylfluoride, and pantetheine from Calbiochem; 1-^{14}C-acetyl-CoA and 1,3-^{14}C-malonyl-CoA from New England Nuclear; *N*-ethylmaleimide from Pierce Chemical Co.; diisopropyl fluorophosphate from Sigma Chemical Co.; pepsin (2 × crystallized) and catalase from Worthington Biochemical Corp.; and egg albumin and human transferrin from Pentex Biochemicals. Iodoacetamide was purchased from General Biochemicals and was recrystallized from a mixture of ethylacetate and pentane before use. *N*,*N'*-Diacetyl cystamine and di-*N*-(*N*-acetyl-β-alanyl)-cystamine were provided by Dr. H. Schulz. All the disulfide compounds were reduced with dithiothreitol (1.1 equiv/equiv were used, and the mixture was incubated for 10 min at 37°), prior to use as substrates.

Malonyl-CoA was synthesized by the method of Eggerer and Lynen (10), its purity being established as described previously (11). Octanoyl-CoA was prepared via the corresponding acid chloride by the method of Seubert (12). ACP was purified from a crude acid precipitate by an earlier procedure (13).

Synthesis of malonyl-ACP. Malonyl-ACP was chemically synthesized by transesterification between *S*-malonyl-*N*-caprylcysteamine and reduced ACP. *S*-Malonyl-*N*-caprylcysteamine was synthesized by the method of Eggerer and Lynen (10). The transesterification was carried out as follows: A sample (1 ml) of a mixture containing reduced ACP (10 μmoles), *S*-malonyl-*N*-caprylcysteamine (40 μmoles), and potassium bicarbonate (330 μmoles) was incubated in a sealed flask for 1 hr at room temperature (25°). The mixture was then continuously extracted in a liquid–liquid extractor for 1 hr with freshly distilled diethyl ether in order to remove the *N*-caprylcysteamine formed during the reaction. The aqueous solution was acidified with dilute HCl to pH 1, and the mixture was extracted with diethyl ether for another hour. The ether was removed and the solution was readjusted to pH 5.0 with $KHCO_3$. The resulting solution was assayed for malonyl-ACP with the fatty acid synthetase of pigeon liver, as described by Plate *et al.* (9). The yield of malonyl-ACP was 70%.

1,3-^{14}C-malonyl-ACP (2.17 μCi/μmole) was prepared enzymatically with *E. coli* malonyltransacylase. The reaction mixture contained potassium phosphate, pH 6.5, 0.05 M; dithiothreitol, 0.01 M; ACP, 0.2 mM; 1,3-^{14}C-malonyl-CoA, 2.35 mM; and malonyltransacylase, 4.8 units (see below), in a final volume of 10 ml. After incubation of the mixture for 30 min at 25°, the reaction was stopped by the addition of 1 ml of 25% trichloroacetic acid. The precipitated protein was collected by centrifugation, washed successively with ethanol and ether, and dried in air. The solid residue was dissolved in water and the pH was adjusted to 5.

Assay of malonyltransacylase. The enzyme was assayed essentially according to the method of Plate *et al.* (9). A typical reaction mixture contained potassium phosphate, pH 6.5, 0.05 M; dithiothreitol, 10 mM; ACP, 75 μM; malonyltransacylase (0.2–1 munit) and 1,3-^{14}C-malonyl-CoA (2 μCi/μmole), 100 μM in a final volume of 0.2 ml. All the components of the reaction mixture, except 1,3-^{14}C-malonyl-CoA, were preincubated for 10 min at 25°. The reaction was started by the addition of 1,3-^{14}C-malonyl-CoA. After incubation for 2 min at 25°, the reaction was stopped by the addition of 0.5 ml of cold 5% perchloric acid. The collection of acid-precipitable radioactivity on Millipore filters (25-mm diameter, pore size 0.45 μ) and the subsequent washing and counting of the filters were carried out as described by Williamson and Wakil (7). Under these conditions, the incorporation of radioactivity into ACP was linear up to 5 min and was proportional to the amount of enzymic protein. Blank reactions without the enzyme and containing the same amounts of 1,3-^{14}C-malonyl-CoA and ACP as the experimental samples, were always carried out. The radioactivity present in the blank samples was less than 4% of that in the experimental samples, and proper corrections were made for the experimental samples by subtracting the blank values. One unit of enzyme was defined as the amount of protein required to catalyze the transacylation of 1 μmole of malonyl group per minute. Specific activity was defined as units per mg protein. Protein was measured by the biuret method (14).

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caprylcysteamine formed during
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o pH 1, and the mixture was ex-
hyl ether for another hour. The
l and the solution was readjusted
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When malonyltransacylase was assayed in the reverse direction, the above procedure was employed except that CoA (50 μM) was used instead of ACP, and the reaction was started with the addition of 1,3-^{14}C-malonyl-ACP (20 μM). The loss in acid-precipitable radioactivity was taken to represent the radioactive malonyl groups transacylated to the acceptor substrate. In the absence of enzyme, there was no transfer of the malonyl group from ^{14}C-malonyl-ACP to other thiol acceptors.

Determination of malonyl-enzyme intermediate. Malonyl binding to the enzyme was carried out as described earlier (8). Routinely, the reaction mixture contained potassium phosphate, pH 6.5, 100 mM; dithiothreitol, 10 mM; 1,3-^{14}C-malonyl-CoA (10.4 μCi/μmole), 96 μM; and malonyltransacylase, 0.2 to 1.0 unit, in a final volume of 0.2 ml. Incubation was carried out at 25°. The reaction was started with the addition of malonyl-CoA and stopped after 2 min with the addition of 0.05 ml of 25% trichloroacetic acid. At this stage, 1.0 mg of protein from the 55–85% ammonium sulfate saturation fraction, as obtained in the purification procedure described later, was added as carrier to reaction mixtures which contained less than 1 mg of malonyltransacylase. The resulting protein precipitate was collected on glass fiber filters (Whatman GF/C, 24 mm in diameter), and the filters were then washed and counted as described previously (8).

Preparation of ^{14}C-malonyl-enzyme. A reaction mixture contained potassium phosphate (pH 6.5), 50 mM; dithiothreitol, 1 mM; 1,3-^{14}C-malonyl-CoA (10.4 μCi/μmole), 96 μM; and malonyltransacylase (sp act, 157 units/mg), 2.8 mg, in a final volume of 0.4 ml. The reaction was started by the addition of ^{14}C-malonyl-CoA and the mixture was incubated for 2 min at 25°. The solution was then applied to a Sephadex G-50 column (1.1 × 27 cm) which had been equilibrated with 0.05 M potassium phosphate (pH 6.5). The column was eluted with the same phosphate buffer, and 1-ml fractions were collected. The protein emerging at the void volume contained ^{14}C-malonyl group and separated well from the ^{14}C-malonyl-CoA.

Performic acid oxidation. Performic acid oxidation was carried out according to the method of Hirs (15). Experimental details are described in our earlier publication (8). Thioesters are oxidized by performic acid to their corresponding carboxylic acids and sulfonic acids (16). Hence, performic acid oxidation was routinely used to measure the amount of acyl groups covalently bound to thiol and nonthiol groups (8, 17).

Peptic digestion of malonyl-enzyme intermediate. Large-scale incubation (17-fold over the routine assay) of malonyltransacylase with 1,3-^{14}C-malonyl-CoA was performed as described in "Determination of Malonyl-Enzyme Intermediate." The washing of the precipitated ^{14}C-malonyl protein and its peptic digestion was carried out as described previously (8).

Fractionation of ^{14}C-malonyl peptides by ion-exchange chromatography. The ^{14}C-malonyl peptides were fractionated on a Dowex 50-X8 column (0.9 × 17 cm) with gradient elution with volatile pyridine–acetic acid buffers, as previously described (8). Three different peptides fractions containing radioactivity were obtained (PM1, PM2, and PM3).

Inactivation of malonyltransacylase by phenylmethanesulfonylfluoride. Phenylmethanesulfonylfluoride (50 mM in isopropanol) was added to malonyltransacylase (0.065 mg in 1 ml of 0.01 M potassium phosphate, pH 7.4) to final concentrations of 0.1, 1.0, and 3 mM. The mixtures were incubated at 25° for 60 min and then diluted 2500-fold with 0.01 M potassium phosphate, pH 7.4. Aliquots of each mixture (0.01 ml) were assayed for transacylase activity as described under "Assay of Malonyltransacylase." Isopropanol at the levels used in these experiments had no effect on the enzymic activity.

Chromatography of 1,3-^{14}C-malonyl-CoA, 1,3-^{14}C-malonyl-pantetheine and 1,3-^{14}C-malonyl-N-acetyl-cysteamine. In malonyltransacylase assays where these derivatives were studied, the following procedure was used: After 2 min of incubation, the reaction was stopped by the addition of 0.01 ml of 6 N HCl. The sample was dried, in vacuo, and the residue was dissolved in 0.05 ml of water. An aliquot was chromatographed on Eastman chromagram thin-layer cellulose sheets in an ascending system of isobutyric acid:concentrated NH_4OH:water (66:1:30). Malonyl-CoA (R_F = 0.44) separated well from malonyl-pantetheine or malonyl-*N*-acetyl-cysteamine (R_F = 0.75). The chromagrams were cut into 1-cm strips and the radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer.

Assay of E. coli fatty acid synthetase. The fatty acid synthetase from *E. coli* was assayed by the method of Pugh *et al.* (18). Incubations were performed for 30 min at 37°. Before saponification, palmitic acid (0.5 mg) in ethanol was added to each sample as carrier.

Disc-gel electrophoresis. Acrylamide disc-gel electrophoresis was carried out according to the method of Davis (19). Gels of 7.5% acrylamide (5 × 60 mm) were run at a constant current of 1 mA per gel at room temperature with an electrode buffer of Tris–glycine (pH 8.9). Samples containing sucrose and tracking dye (bromophenol blue) were layered directly onto the spacer gel. When the gels were assayed for transacylase ac-

tivity, they were rapidly frozen and sliced into cross sections 1 mm thick. Each section was eluted with 0.5 ml of 0.01 M potassium phosphate (pH 7.4) for 1 hr at 4° and assayed for malonyltransacylase activity.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis was carried out on a 10% acrylamide gel according to the method of Weber and Osborn (20). Stained gels were scanned for absorbance with a model 2410 Linear transport attached to a Gilford model 240 spectrophotometer.

Preparation of malonyltransacylase. Malonyltransacylase from *E. coli* was purified by a method similar to that of Williamson and Wakil (7). All operations for enzyme purification were performed at 4°. All buffers, except that in which the final enzyme was stored, contained 0.005 M β-mercaptoethanol. Frozen cells of *E. coli* E-26 (500 g, wet weight) were suspended in 1 liter of 0.01 M potassium phosphate (pH 7.4) and sonically irradiated (in 100-ml batches) for 7 min with a Branson sonifier at a temperature not exceeding 5°. The resulting suspension was centrifuged at 39,000*g* for 30 min, and the supernatant solution (crude extract) was decanted. Solid ammonium sulfate was added to this solution to 55% saturation. The resulting precipitate was removed by centrifugation and the supernatant solution was then adjusted to 85% saturation by further addition of solid ammonium sulfate. The precipitated protein was collected by centrifugation, and the supernatant fluid was discarded. The precipitate was dissolved in 0.01 M potassium phosphate buffer (pH 7.4) and dialyzed overnight against the same buffer.

The dialyzed solution, as obtained above from two 500-g batches of cells, was applied to a DEAE–cellulose column (6.5 × 60 cm) which had been equilibrated with 0.01 M potassium phosphate (pH 7.4). The column was washed with the same buffer until no further 260 nm-absorbing material was eluted and then rewashed with 4 liters of 0.075 M NaCl in 0.01 M potassium phosphate buffer (pH 7.4). Both of the eluates were discarded. The column was then eluted with a linear gradient made from 4 liters of 0.075 M NaCl in 0.01 M potassium phosphate (pH 7.4) and 4 liters of 0.45 M NaCl in 0.01 M potassium phosphate (pH 7.4). Fractions of 30 ml were collected and assayed for malonyltransacylase activity. The fractions (numbered 160–245) containing high activity were pooled and ammonium sulfate was added to 95% saturation. The precipitated protein was collected by centrifugation and redissolved in a minimum volume of 0.01 M potassium phosphate buffer (pH 7.4).

The protein solution was then applied to a Sephadex G-100 column (5 × 90 cm), equilibrated with 0.01 M potassium phosphate buffer (pH 7.4). The column was eluted with the same buffer, and fractions of 13 ml were collected. The fractions (numbered 77–89) containing malonyltransacylase activity were pooled, and the resulting solution was applied directly to a DEAE–cellulose column (2.4 × 23 cm) which was previously equilibrated with 0.01 M potassium phosphate buffer (pH 7.4). The column was washed first with 0.01 M phosphate (pH 7.4) until no further 260 nm-absorbing material was eluted, and then with 600 ml of 0.1 M NaCl in 0.01 M potassium phosphate (pH 7.4). Both eluates were discarded. The enzyme was then eluted with 800 ml of a linear gradient of 0.1–0.45 M NaCl in 0.01 M potassium phosphate (pH 7.4). Fractions containing high malonyltransacylase activity were pooled, and solid ammonium sulfate was added to 95% saturation. The precipitated protein was collected by centrifugation and redissolved in a minimum volume of 0.01 M potassium phosphate (pH 7.4).

The enzyme solution was applied to a Sephadex G-100 column (2.5 × 40 cm) which had been equilibrated with 0.01 M potassium phosphate (pH 7.4). The column was eluted with the same buffer and 4-ml fractions were collected. Fractions (numbered 24–28) containing high malonyltransacylase activity were pooled, and ammonium sulfate was added to 95% saturation. The precipitated protein was collected by centrifugation and redissolved in 0.05 M potassium phosphate (pH 6.5) containing 1 mM dithiothreitol. The enzyme preparation thus obtained was stored in this buffer at −20°.

A sample of this preparation containing 6 mg of protein was subjected to isoelectric focusing in a 110-ml capacity ampholine column (LKB 8101). Carrier ampholyte (pI 3–6) was used at a concentration of 1% and the density gradient was prepared manually. With cathode at the top of the column, a constant potential of 450 V was applied across the electrodes for 48 hr. At the end of electrofocusing, 1-ml fractions were collected, and the pH and malonyltransacylase activity of each fraction were determined. Fractions containing malonyltransacylase activity were pooled and diluted with an equal volume of 0.01 M potassium phosphate (pH 7.4). Ammonium sulfate was added to 95% saturation; the precipitated protein was collected by centrifugaion, redissolved in a minimum volume of 0.05 M potassium phosphate (pH 6.5) containing 1 mM dithiothreitol and stored at −20°.

RESULTS

Purification and physical properties of malonyltransacylase. A typical purification

TABLE I

PURIFICATION OF MALONYLTRANSACYLASE[a]

Fraction and step	Protein (mg)	Specific activity (μmoles/min/mg)	Total activity (μmoles/min)	Recovery (%)
Crude extract	49680	0.69	34250	100[b]
Ammonium sulfate fractionation (55-85%)	16000	1.50	24000	70
DEAE-cellulose column I	1505	11.15	16800	49
Sephadex G-100 column I	314	42.6	13400	39.1
DEAE-cellulose column II	77.6	129.5	10050	29.3
Sephadex G-100 column II	22.3	342.8	7650	22.3
Electrofocusing (pH 3-6)	(1.5)[c]	653.0	(980)[c]	8.5

[a] The details of the purification scheme are described in "Methods."

[b] Arbitrarily taken as 100.

[c] A sample containing 6 mg of protein from Sephadex G-100 Column II step was used for the electrofocusing.

scheme for malonyltransacylase is shown in Table I. The enzyme was purified about 950-fold, with a yield of 8.5%. The inclusion of the electrofocusing step in this procedure was necessary for the isolation of a homogeneous preparation of enzyme, even though about 55% of the total enzymic activity was lost by this manipulation. The isoelectric point of the malonyltransacylase, measured by the electrofocusing technique, was 4.45. Highly purified preparations of the transacylase were completely inactive after heating at 60° for 20 min, whereas at cruder levels this enzyme has been reported to be heat stable (4, 7).

Disc-gel electrophoresis of the purified transacylase revealed a single band of protein running very near the dye front, indicating the acidic nature of the protein (Fig. 1). Assays for malonyltransacylase activity in eluants from sectioned disc gels showed that the transacylase and the stained protein band had identical mobilities. Sodium dodecylsulfate-polyacrylamide gel electrophoresis showed a single stained band with a molecular weight of 35,500 (Fig. 2). Application of Squire's equation (21) to the elution of malonyltransacylase from Sephadex G-100 column gave an estimated molecular weight of 40,000. The similar molecular weights obtained by gel filtration and sodium dodecylsulfate-polyacrylamide gel electrophoresis suggested that *E. coli* malonyltransacylase may have no subunit structure.

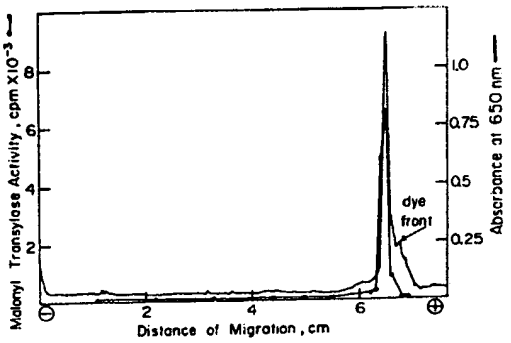


FIG. 1. Activity profile and absorbance scan of malonyltransacylase on acrylamide disc gels. Samples (24 μg protein per gel) of malonyltransacylase (sp act 653 μmoles/min/mg) were layered on the spacer gel. After electrophoresis one gel was sectioned, and the enzyme was eluted and assayed as described in Methods. Stained gel was scanned for absorbance at 650 nm as described in Methods.

Identification of the product of transacylation. The identity of malonyl-ACP as the reaction product of malonyltransacylase was established by the same procedures reported earlier (9): (a) The acid-precipitable radioactive product cochromatographed with ACP on a DEAE-cellulose column, suggesting that the product was acyl-ACP. (b) To establish that the malonyl groups were not modified during their incubation with malonyltransacylase, the acid-precipitable radioactive product was treated with neutral hydroxylamine (22) and the resulting hydroxamate chromatographed on silica gel thin-

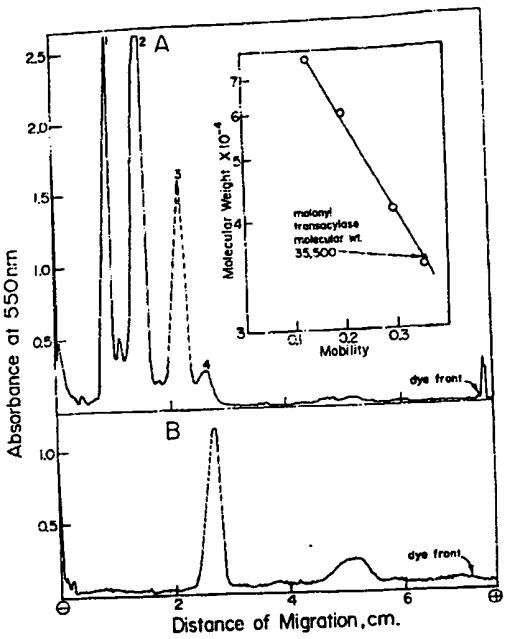


FIG. 2. Determination of molecular weight of malonyltransacylase by sodium dodecylsulfate–polyacrylamide gel electrophoresis. Protein samples were prepared according to the method of Weber and Osborn (21). Sample A contained 20 μg each of human transferrin, catalase, and egg albumin and 4 μg of pepsin. Sample B contained 20 μg of malonyltransacylase (sp act 653 μmoles/min/mg). Scanning of gels was performed as described in Methods. The small broad peak in Fig. 2B with a migration distance of 5.1 cm was presumably due to the ampholytes present in the sample, since a sample obtained from a blank run of isoelectric focusing without any protein gave an identical band with the same migration distance. The peaks 1, 2, 3, and 4 in Fig. 2A are due to human transferrin, catalase, egg albumin, and pepsin, respectively. The insert in Fig. 2A shows the relative mobilities of various proteins with respect to their molecular weights. The relative mobility of malonyltransacylase is indicated by x.

layer sheet (23). The product of malonyltransacylase yielded a radioactive hydroxamate which cochromatographed (R_F = 0.03) with an authentic malonyl hydroxamate. (c) When the radioactive acid-precipitable product of malonyltransacylase was incubated with acetyl-CoA, TPNH, and *E. coli* fatty acid synthetase, the radioactivity was incorporated into fatty acids, indicating the identity of malonyl groups throughout their incubation with malonyltransacylase. (d) Finally, performic acid oxidation of the acid-precipitable product resulted in the loss of all acid-precipitable radioactivity, indicating that the malonyl group was attached to the ACP through a thioester linkage.

Covalent binding of malonyl group to malonyltransacylase. Earlier studies with the pigeon liver fatty acid synthetase suggested the participation of nonthiol groups (hydroxyl group of serine) in the acetyl- and malonyltransacylation reactions of this complex (8, 9). The isolation of a homogeneous preparation of malonyltransacylase from *E. coli* afforded an opportunity to test whether a nonthiol group was also involved in the reaction catalyzed by this enzyme. As can be seen in Table II, malonyl group binds to the transacylase at all stages of purification. That this binding occurs at a nonthiol site is evident from its stability to performic acid oxidation. Over 90% of the ^{14}C-malonyl group bound to the enzyme was recovered with the protein after performic acid oxidation. Moreover, the amount of malonyl groups bound per unit enzyme remained constant throughout the purification of the transacylation (Table II), suggesting that this binding is an inherent property of the transacylase.

Digestion of the ^{14}C-malonyl-enzyme with pepsin and subsequent fractionation on Dowex 50-X8 column (see Methods) yielded three different radioactive peptides—PM1, PM2, and PM3. As shown in Table III, the ^{14}C-malonyl groups bound to all three peptic peptides were stable to performic acid oxidation indicating that these groups were covalently bound through a nonthioester linkage.

To determine whether this nonthiol site functions in the transacylase catalysis, the following experiment was performed: ^{14}C-malonyl-enzyme was prepared and the transfer of the ^{14}C-malonyl group to ACP or CoA was followed. As shown in Table IV, the addition of ACP or CoA to the ^{14}C-malonyl-enzyme resulted in the loss of 43 and 66% of the radioactivity stable to performic acid oxidation, respectively, indicating that the malonyl-enzyme can donate the malonyl group to either substrate, i.e., ACP or CoA.

TABLE II

COVALENT BINDING OF MALONYL GROUPS TO MALONYLTRANSACYLASE AT VARIOUS STAGES OF PURIFICATION

Steps	Specific activity (μmoles/min/mg)	Total malonyl bound (nmoles/mg protein)	Malonyl bound at nonthiol site (nmoles/mg protein)	Malonyl bound at nonthiol site (nmoles/unit enzyme[a])
DEAE-cellulose column I	8.15	0.246	0.185	0.0226
Sephadex G-100 column I	86.30	1.570	1.445	0.0167
DEAE-cellulose column II	98.80	1.690	1.506	0.0153
Sephadex G-100 column II	200.00	3.090	2.808	0.0144
Isoelectric focusing	520.00	8.020	7.57	0.0145

[a] A unit of enzyme is the amount of enzyme which catalyzes the transacylation of 1 μmole of malonyl groups per minute.

TABLE III

STABILITY OF ^{14}C-MALONYL PEPTIC PEPTIDES TO PERFORMIC ACID OXIDATION[a]

^{14}C-Malonyl peptides	Radioactivity stable to performic acid oxidation (%)
PM1	88
PM2	99
PM3	99

[a] Peptic peptide fractions PM1, PM2, and PM3 were obtained by ion-exchange chromatography as described in Methods. Performic acid oxidation was carried out as described in Methods. After performic acid oxidation, aliquots of the samples were applied to Whatman glass fiber disc (GF/C, 24 mm), and the discs were dried under infrared lamp. The discs were then washed six times with 5 ml each of anhydrous diethyl ether, and the radioactivity in the discs was measured in a Packard Tri-Carb scintillation spectrometer.

Inactivation of malonyltransacylase by phenylmethanesulfonylfluoride. Phenylmethanesulfonylfluoride (PMSF), a compound which inactivates many of the serine proteases by sulfonylation of the hydroxyl group of a specific serine residue in the active site (24), was tested for its effect on malonyltransacylase. As shown in Table V, PMSF at a concentration of 1 mM inactivated the enzyme by 91%, suggesting that the transacylase has an active serine. Incubation of the malonyltransacylase with malonyl-CoA resulted in protection of the enzyme against PMSF inactivation, indicating that the two reagents were reacting with the enzyme at the active site. Coenzyme A, acetyl-CoA, or octanoyl-CoA also protected the transacylase against inactivation by PMSF, as shown in Table V.

TABLE IV

ABILITY OF ACP AND COENZYME A TO SERVE AS ACCEPTORS OF THE MALONYL GROUP FROM ^{14}C-MALONYL-ENZYME[a]

Addition to the acyl-enzyme	Covalently bound malonyl groups: Total (nmoles)	Nonthiol site (nmoles)	Nonthiol site: % Loss of ^{14}C-malonyl
None	0.285	0.290	0
ACP	0.250[b]	0.165	43
CoA	0.098	0.099	66

[a] ^{14}C-Malonyl-enzyme was prepared as described in Methods. ^{14}C-Malonyl-enzyme (0.4 mg) was incubated for 5 min at room temperature with ACP (0.167 mM) or CoA (0.334 mM) in a final volume of 1.5 ml. The reaction was stopped by the addition of 0.2 ml of 25% trichloroacetic acid. Determination of covalently bound malonyl groups before and after performic acid oxidation was carried out as described in Methods. The values given are for ^{14}C-malonyl bound to 0.16 mg of enzymic protein.

[b] The total ^{14}C-malonyl group bound to protein in this experiment represents that which is bound to ACP and the transacylase.

Diisopropylfluorophosphate, another inactivator of serine proteases (25), at concentrations of up to 3 mM, did not inhibit malonyltransacylase. Neither did *N*-ethylmaleimide or iodoacetamide inhibit the transacylase even at inhibitor concentrations of up to 10 mM. Moreover, these inhibitors had no effect on the formation of malonyl-enzyme intermediate from malonyl-CoA. These results strongly indicate that the enzyme is not a sulfhydryl enzyme, contrary to earlier reports (7, 26) based upon data

TABLE V

EFFECT OF PHENYLMETHANESULFONYLFLUORIDE (PMSF) ON THE MALONYLTRANSACYLASE[a]

PMSF concentration (mM)	Preincubation with	Inhibition (%)
0.1		18
1.0		91
3.0		100
1.0	Malonyl-CoA (30 μM)	93
1.0	Malonyl-CoA (90 μM)	14
1.0	Malonyl-CoA (180 μM)	0
1.0	CoA (90 μM)	17
1.0	Acetyl-CoA (90 μM)	49
1.0	Octanoyl-CoA (95 μM)	62

[a] Incubation of the enzyme with PMSF and its subsequent assay were performed as described in Methods. In experiments where acyl-CoA's were used, the enzyme was incubated with the acyl-CoA at the indicated concentration at 25° for 5 min and then treated with PMSF.

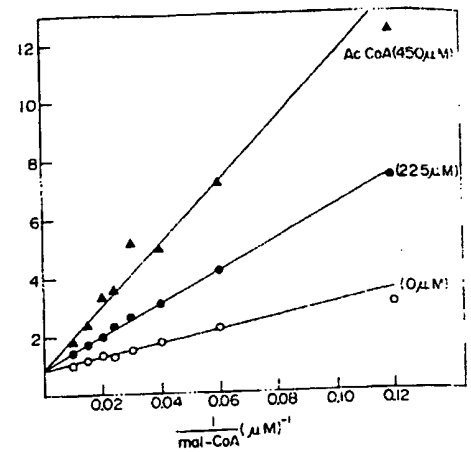


FIG. 3. The rate of malonyltransacylation as a function of malonyl-CoA concentration at various levels of acetyl-CoA. The data are plotted on reciprocal coordinates. Malonyltransacylase activity was assayed as described in Methods, except that the indicated concentrations of 1,3-^{14}C-malonyl-CoA and acetyl-CoA were used. Units on the ordinate are (nmoles of malonyltransacylated per min)$^{-1}$.

from relatively crude preparations of the enzyme.

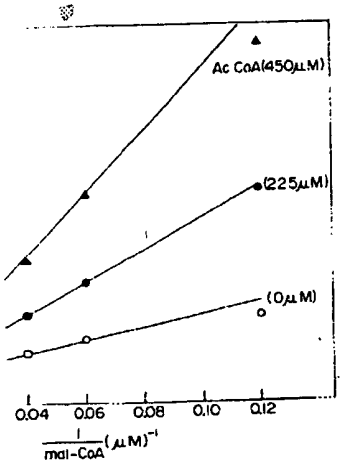
Specificity of malonyltransacylase. It has been shown earlier (4, 7, 26) that malonyltransacylase is specific for the reversible transfer of malonyl group but not of acetyl group from CoA to ACP. The fact that the transacylase does not form acetyl-enzyme after incubation with acetyl-CoA indicates that the formation of acyl-enzyme intermediate is also specific for the malonyl group. Acetyl-CoA, however, inhibited the malonyltransacylase reaction, and this inhibition was competitive with malonyl-CoA as shown in Fig. 3. A K_i value of 115 μM was calculated for this inhibition. The K_m value for malonyl-CoA was found to be 25 μM.

Although malonyltransacylase is specific for the malonyl group, it shows broader specificity with regard to the thiol acceptors. In the presence of malonyl-CoA, the enzyme was able to transfer the malonyl group from CoA to ACP, pantetheine, or *N*-acetylcysteamine. The kinetic parameters (K_m and V_{max}) of the reaction in both the forward and reverse directions were determined using various thiol acceptors (Table VI). ACP or CoA were the preferred acceptors for the malonyl group from malonyl-CoA or malonyl-ACP, respectively. Other thiol acceptors (pantetheine, *N*-acetylcysteamine, or *N*-(*N*-acetyl-β-alanyl)-cysteamine) also accept the malonyl group but only at relatively higher concentrations (Table VI). The rates of transacylation at various concentrations of the thiol acceptor were measured and the K_m and V_{max} values calculated from Lineweaver–Burk plots. While these values were useful for the study of overall specificity of the transacylase, the K_m values obtained for the different acceptors do not indicate the affinity of the enzyme for these compounds since the K_m values in these cases are kinetic quantities rather than true equilibrium dissociation constants (27).

Kinetic studies of malonyltransacylase. Several kinetic parameters of the malonyltransacylase reaction were investigated to provide some insight into the mechanism of this reaction. When the rate of the reaction malonyl-CoA + ACP → malonyl-ACP + CoA was measured at various malonyl-CoA concentrations and at several fixed concentrations of ACP, and the data were plotted on reciprocal coordinates, a series of parallel lines typical for Ping-Pong kinetics (28) were obtained (Fig. 4A). Replots of inter-

Exp | WR
mal CoA 25 μM | 40 μM
ACP 54 μM | 40–100 μM
20 μM

Aro



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etheine, *N*-acetylcysteamine, yl-β-alanyl)-cysteamine) also alonyl group but only at rela- centrations (Table VI). The tion at various concentra- thiol acceptor were measured nd V_{max} values calculated from Burk plots. While these values r the study of overall specificity ylase, the K_m values obtained ent acceptors do not indicate f the enzyme for these com- the K_m values in these cases quantities rather than true issociation constants (27).

udies of malonyltransacylase. ic parameters of the malonyl- reaction were investigated to insight into the mechanism of When the rate of the reaction + ACP → malonyl-ACP + sured at various malonyl-CoA s and at several fixed concen- P, and the data were plotted coordinates, a series of parallel for Ping-Pong kinetics (28) d (Fig. 4A). Replots of inter-

TABLE VI

THE ACYLATION OF VARIOUS THIOLS BY MALONYLTRANSACYLASE[a]

Thiol acceptors of malonyl group from: Malonyl-CoA	Malonyl-ACP	K_m (M)	V_{max} (μmoles/min/mg protein)
ACP		5.4×10^{-5}	572
Pantetheine		3.2×10^{-2}	1245
N-Acetylcysteamine		48×10^{-2}	286
	CoA	2.8×10^{-5}	2940
	Pantetheine	6.0×10^{-5}	1740
	N-(*N*-acetyl-β-alanyl)-cysteamine	3.3×10^{-2}	3820
	N-Acetylcysteamine	17.0×10^{-2}	14300

[a] The malonyltransacylase assays were carried out as described in Methods except that several concentrations of the thiol acceptors were used. In the experiments where malonyl-CoA and pantetheine or *N*-acetylcysteamine were used, the malonyl pantetheine and the malonyl *N*-acetylcysteamine were separated by thin-layer chromatography as described in Methods. The K_m and V_{max} values were calculated from Lineweaver–Burk plots.

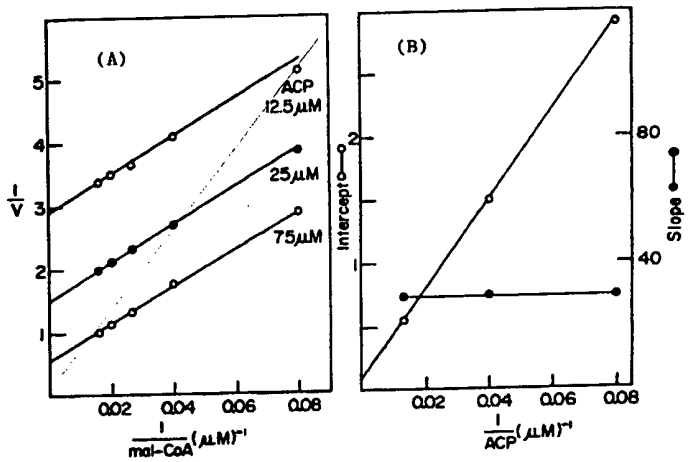


FIG. 4. The rate of malonyltransacylation as a function of malonyl-CoA concentration at several fixed concentrations of ACP. A. Data are plotted on reciprocal coordinates. Malonyltransacylase activity was measured as described in Methods, except that the indicated concentrations of ^{14}C-malonyl-CoA and ACP were used. Units on the ordinate are (nmoles of malonyltransacylated per min)$^{-1}$. B. A replot of the intercepts obtained from Fig. 4A against reciprocal of concentration of ACP. An enzyme concentration of 5.98×10^{-10} moles/liter was used in each assay, and one active site per molecular weight of 35,500 was assumed for the calculation of k_{cat}.

cepts against reciprocal concentrations of the nonvaried substrate ACP gave a straight line as illustrated in Fig. 4B. From this plot, a K_m^{∞} value of 400 μM was estimated for ACP at saturating concentration of malonyl-CoA. At V_m^{∞}, the value of the catalytic rate constant of the reaction $k_{cat} = k_2k_4/k_2 + k_4$ was estimated to be 1.58×10^2 sec^{-1}. Similar kinetic studies for the reverse reaction could not be performed because the available preparations of ^{14}C-malonyl-ACP contained appreciable amounts of free ACP, which would render such investigations difficult.

The kinetic scheme below is consistent with the reaction mechanism wherein the reaction proceeds through two half-reactions in which one product dissociates from a covalent malonyl-enzyme intermediate be-

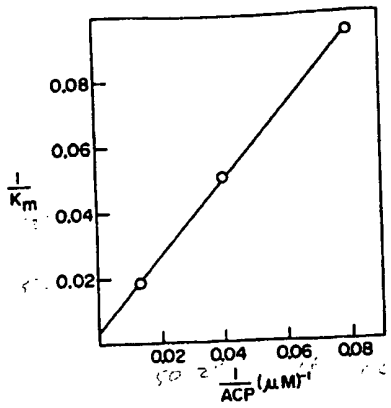


FIG. 5. Relationship between the K_m for malonyl-CoA and ACP concentration. The data are plotted on reciprocal coordinates. Units on the ordinate are $(\mu M)^{-1}$. The K_m values for malonyl-CoA were calculated from Fig. 4A.

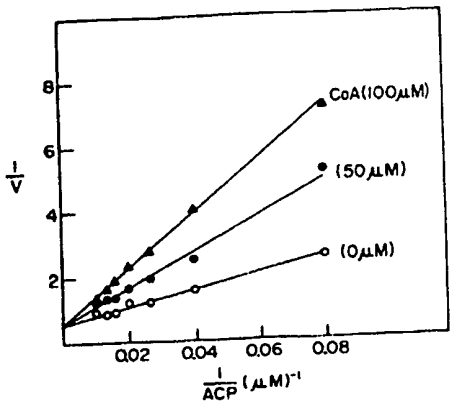


FIG. 6. The rate of transacylation as a function of ACP concentration at different CoA concentrations. The data are plotted on reciprocal coordinates. Malonyltransacylase activity was assayed as described in Methods, except that the indicated concentrations of ACP and CoA were used. Units on the ordinate are (nmoles of malonyl-transacylated per min)$^{-1}$.

fore the second substrate reacts:

$$\text{E} + \text{Mal—CoA} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{E·Mal—CoA} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{E—Mal} + \text{CoA}. \quad (1)$$

$$\text{E—Mal} + \text{ACP} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{E—Mal·ACP} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \text{E} + \text{Mal—ACP}. \quad (2)$$

The steady-state rate equation (29) for the kinetic scheme shown above is

$$\frac{1}{V} = \frac{K_I}{[\text{Mal—CoA}]k_2E_0} + \frac{1}{k_2E_0} + \frac{K_{II}}{[\text{ACP}]k_4E_0} + \frac{1}{k_4E_0}, \quad (3)$$

where $K_I = (k_{-1} + k_2)/k_{-1}$ and $K_{II} = (k_{-3} + k_4)/k_{-3}$. These are the apparent dissociation constants for malonyl-CoA-enzyme complex and malonyl-enzyme-ACP complex, respectively.

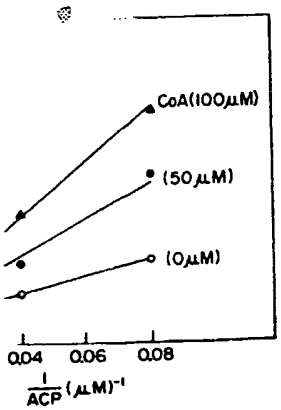
At $-1/K_m$, $1/V = 0$. Thus, Eq. (3) can be rewritten as

$$-\frac{1}{K_m} = \frac{1}{K_I}\left(1 + \frac{k_2}{k_4} + \frac{K_{II}k_2}{k_4[\text{ACP}]}\right). \quad (4)$$

It is apparent from Eq. (4) that a plot of $1/K_m$ versus $1/[\text{ACP}]$ should give a straight line with slope $= K_{II}k_2/K_Ik_4$ and intercept $= 1/K_I + k_2/K_Ik_4$. Such a plot was indeed obtained (Fig. 5) when the K_m values for malonyl-CoA at various nonvaried concentrations of ACP were plotted against ACP concentrations on reciprocal coordinates. From this plot, the value of K_m^∞ for malonyl-CoA at saturating concentration of ACP was calculated to be 290 μM. Lineweaver-Burk plots at varying concentrations of ACP and at several fixed concentrations of the second substrate, malonyl-CoA, also gave a series of parallel lines. The K_m^∞ values calculated for malonyl-CoA and ACP were 210 μM and 301 μM, respectively, values in reasonable agreement with those reported above.

The kinetic parameters of product inhibition of the transacylase reaction were also investigated. As shown in Fig. 6, CoA was a competitive inhibitor of ACP with a K_i value of 45 μM. Malonyl-ACP also inhibited the transacylase reaction. This inhibition was competitive with respect to malonyl-CoA, as shown in Fig. 7. The K_i value for malonyl-ACP was found to be 16 μM.

Equilibrium constant of the malonyltransacylase reaction. The equilibrium constant for the transacylase reaction was reported



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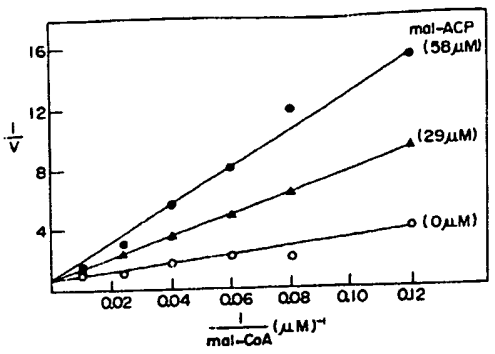


FIG. 7. The rate of transacylation as a function of malonyl-CoA concentration. The data are plotted on reciprocal coordinates. Malonyltransacylase activity was assayed as described in Methods, except that the indicated concentrations of ^{14}C-malonyl-CoA and malonyl-ACP were used. Units on the ordinate are (nmoles of malonyl-transacylated per min)$^{-1}$.

to be about 2.33 (7), based upon measurements made with relatively crude transacylase preparations and malonyl-ACP containing considerable amounts of free ACP. We reinvestigated this problem using ^{14}C-malonyl-CoA, pure ACP, and homogeneous preparations of malonyltransacylase. The reaction was allowed to reach equilibrium, and the reactants were analyzed. The equilibrium constant, determined at four different malonyl-CoA concentrations, was found to be 0.018 ± 0.003. This value is much smaller than that reported earlier (7), indicating that the equilibrium of the transacylase reaction favors malonyl-CoA formation. The equilibrium constant for the formation of malonyl-enzyme, as defined by

$$K^{I} = \frac{[E]\,[Malonyl—CoA]}{[E—Mal]\,[CoA]},$$

was found to be 11.2. The equilibrium constant for the formation of malonyl-enzyme from malonyl-ACP, as defined by

$$K^{II} = \frac{[E]\,[Malonyl—ACP]}{[E—Mal]\,[ACP]},$$

was calculated from the expression $K_{eq} = K^{II}/K^{I}$. By substituting the values for K_{eq} and K^{I} in this equation, a value of 0.20 was obtained for K^{II}, suggesting that the formation of malonyl-enzyme from malonyl-ACP is favored.

DISCUSSION

Homogeneous preparations of malonyltransacylase were prepared from extracts of *E. coli*. The enzyme gave a single band on disc-gel electrophoresis. A molecular weight of 35,500 daltons was estimated from its mobility on disc-gel electrophoresis with sodium dodecyl sulfate. Like ACP (30), malonyltransacylase is an acidic protein with an isoelectric point of 4.45.

During catalysis, malonyltransacylase forms a malonyl-enzyme intermediate in which the malonyl group is covalently bound to the protein in a nonthiol bond, as shown in Eqs. (5) and (6):

$$\text{malonyl-CoA} + \text{E–OH} \rightleftharpoons \text{malonyl-O–E} + \text{CoA}, \quad (5)$$

$$\text{malonyl-O-E} + \text{ACP} \rightleftharpoons \text{malonyl-ACP} + \text{E–OH}, \quad (6)$$

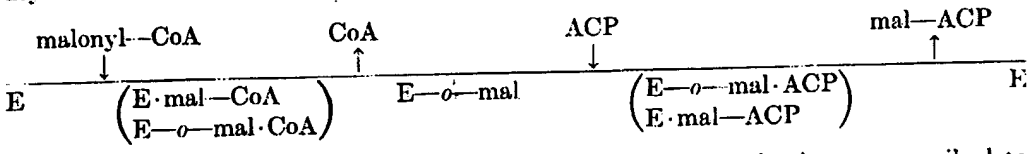
where E–OH represents the enzyme with its nonthiol binding site. This conclusion was based on several observations. An acyl-enzyme was formed from malonyl-CoA and was isolated by precipitation with perchloric acid. The covalently bound malonyl group was stable to performic acid oxidation, suggesting that the malonyl group was bound to the enzyme via a nonthioester linkage. Hydrolysis of the malonyl-enzyme intermediate with pepsin yielded malonyl-peptides which were also stable to performic acid. The malonyl binding at the nonthiol site per unit enzyme was constant throughout the purification of the enzyme, suggesting that the acyl binding was intimately associated with malonyltransacylase. The covalently bound malonyl group could be readily transferred to ACP or CoA on incubation of these substrates with the malonyl-enzyme.

The transacylase was not a sulfhydryl enzyme since it was not inhibited by sulfhydryl-alkylating agents. The enzyme was markedly inhibited by phenylmethanesulfonylfluoride, an inhibitor of several proteases with active serine residue. Malonyl-CoA, CoA, acetyl-CoA, or octanoyl-

CoA protected the enzyme against this inhibition when incubated with the enzyme before interaction with the phenylmethanesulfonylfluoride. It is noteworthy here that the acetyl- and malonyltransacylase activities of the pigeon liver fatty acid synthetase were also inhibited by phenylmethanesulfonylfluoride and that this inhibition was overcome by preincubating the enzymes with acetyl-CoA or malonyl-CoA.[3] Thus, the transacylases of the pigeon liver fatty acid synthetase and the *E. coli* malonyltransacylase must have the same type of nonthiol group at the active center and probably share a common transacylation mechanism. The same mechanism apparently operates in the fatty acyl transferase activity of the yeast fatty acid synthetase where a nonthiol site is involved in the transacylation reaction (31). The nature of the nonthiol group involved in this transacylation is unknown at present. However, the hydroxyl group of a serine appears to be most likely.

Further support for the formation of a malonyl-enzyme intermediate during transacylation was derived from kinetic studies of this reaction. Since a covalent enzyme-substrate intermediate (malonyl-enzyme) was formed during the reaction, the mechanism of the reaction could generally be expected to follow Ping-Pong kinetics (32–34). The initial velociy patterns obtained for the transacylase show that the reaction catalyzed by this enzyme follows the steady-state rate equation (Eq. 3) as evidenced by the parallel lines obtained when $1/V$ is plotted against 1/[malonyl-CoA] at various constant concentrations of ACP. This behavior is expected of the reactions described by Eqs. (1) and (2), in which only binary enzyme substrate complexes are formed; one product dissociates from the enzyme to leave an intermediate form of the enzyme containing part of the other product, and this then reacts with the second substrate to give the final product. If both the substrates had to be bound to the enzyme before release of a product (i.e., a mechanism involving a ternary complex), the initial velocity pattern would be a family of lines intersecting to the left of the vertical axis (28). But no such pattern was obtained. Moreover, the plot of $1/K_m$ versus 1/[ACP] was linear (Fig. 5) as predicted by Eq. (4), thus providing further support for the Ping-Pong mechanism.

The competitive inhibition of ACP by CoA and of malonyl-CoA by malonyl-ACP was consistent with the mechanism expressed in Eqs. (1) and (2). The competitive inhibition by CoA of the acylation of ACP by malonyl-CoA was obvious, since each molecule of malonyl-enzyme formed from malonyl-CoA may react either with ACP to give malonyl-ACP or with CoA to resynthesize malonyl-CoA. In a similar way the competitive inhibition of malonyl-CoA by malonyl-ACP may also be explained. Therefore, the kinetics of the reaction catalyzed by malonyltransacylase, (i.e., initial velocity and product inhibition) are consistent with a Ping-Pong Bi Bi mechanism (28):



The Ping-Pong mechanism was ascribed to other enzymic reactions among them glutamic oxaloacetic transaminase (32), coenzyme A transferase (33), nucleoside diphosphokinase (35), phosphoglucomutase (36), sucrose phosphorylase (37), and a number of flavin enzymes wherein hydrogen is transferred as a part of the bound reduced coenzyme (38).

The available data did not allow us to calculate the individual rate constants of the reaction. The only constant that could be determined from the experimental data was the catalytic rate constant (k_{cat}), which is made of several rate constants. The value for this constant, 1.58×10^3 sec^{-1}, is of the same order of magnitude as the k_{cat} value estimated for the reaction catalyzed by coenzyme A transferase (33).

Malonyltransacylase was specific for the

[3] V. C. Joshi, unpublished observation.

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ACKNOWLEDGMENT

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CoA (uM)	0	0.8	2.0	5.0	12.0	30.0	100.0
Data (cpm)	866	783	586	448	223	143	62